# A LITERATURE REVIEW OF THE STABILITY OF STORED NUTRITIONAL AND STEROID HORMONE BIOMARKERS

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Epidemiology – Master of Science

2013

ABSTRACT

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Archiving and storage of biological materials for use in research has seen a significant upswing in interest and activity in the last decade and will likely continue to grow as a research resource and an industry. Despite all of this activity and economic outlay there is a fundamental question that remains unanswered regarding the storage of biological materials: At what storage temperature does the least amount of degradation occur? Conventional wisdom would suggest that at least -80° C is required for long term storage, however robust evidence is lacking to support -80° C or below as the appropriate long term storage temperature for *all* biological molecules of interest to researchers.

The purpose of this thesis is to review the -20° C and -80° C storage temperature literature for nutritional and steroid hormone analytes and to begin the process of making a scientific case for the appropriate storage temperatures for these analytes. There are limited true validation studies of nutritional and steroid hormones temperature requirements from which to draw robust conclusions. Thus, well-designed storage temperature validity studies are required to ensure that these analytes are stored at a temperature that reduces degradation and ensures that studies that store or use these analytes can be assured that their findings are indeed valid.

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# KEY TO SYMBOLS OR ABBREVIATIONS

C – Celsius

° - Degree

(-) - Negative

ISBER – International Society for Biological and Environmental Repositories

EPIC – European Prospective Investigation into Cancer and Nutrition

NCPP – National Collaborative Perinatal Project

JACC – Japan Collaborative Cohort

NHANES – National Health and Nutrition Examination Survey

FMC – Finnish Maternity Cohort

AA – Ascorbic Acid

DTT – Dithiothreitol

MPA – Metaphosphoric Acid

TAA – Ascorbic Acid and Dehydroascorbic Acid

5mTHF – 5-methytetrahydrofolate

pABG – Amniobenzoylglutamate

B1 – Thiamine

B2 – Riboflavin

B6 – Pyridoxine

B12 – Cyanocoblamin

CVD – Cardiovascular Disease

SHBG – Sex Hormone Binding Globulin

CBG – Corticosteroid Binding Globulin

# INTRODUCTION

The core of this review is storage stability of biospecimens and why this topic matters to epidemiology. The problem of biospecimen storage dates to the 1950s, when some of the early epidemiological studies began archiving biological specimens, generally at -20° Celsius (C). One of the early cohorts that stored biological specimens was the National Collaborative Perinatal Project (1959-1966), and since that time advances in molecular epidemiology have led to the archiving of biological samples at the inception of an increasing number of cohort studies, and as an addition to the protocol in several established longitudinal cohorts such as the Framingham Heart Study. (2, 52) Through these decades, millions of human specimens have been collected and stored in freezers at a huge cost, yet there is a fundamental question that remains unanswered: At what storage temperature is the greatest stability achieved for a given analyte?

While the last two decades have witnessed a significant upswing in archiving interest and activity the answer to the storage temperature question does not seem high on investigators' agenda. (19, 27, 38, 54, 66, 83) The world has witnessed the launching of the UK BioBank, a world class facility intended to receive approximately 19,000 specimens a day or a total of 15 million specimens in over 4 years at a cost of many millions. (23) Great care was taken by the UK BioBank investigators and planners to pilot test all steps in the collection and processing chain, but no mention has been made of pilot testing of the temperature requirements of specimens. (6, 22, 23, 39, 61, 70)

The International Society for Biological and Environmental Repositories (ISBER) in 2012 published "*Best Practices for Repositories"*, but that document too only lists a few references for temperature needs of analytes. (67) Hundreds of articles present investigations of

the roles of biomarkers in disease, but most address specimen stability in a similarly cursory way, with, at most, references to the work of other investigators with the same stored analyte, but rarely with any validation of the measures obtained after storage. The expectation that best-practice protocols for storage of individual analytes would have been formulated, or at least that studies that include a particular biomarker would pilot storage protocols, is nearly always disappointed.

Without a validated storage temperature it is difficult to assure that quality data is contributing to the analytic model. (40, 52, 87) Analyte stability influences every stage of a study using biomarkers, including specimen collection, processing, storage temperature and length, measurement, and even cost. (34, 47, 87) In fact, investigators risk "garbage in, garbage out" if they fail to attend to all that influences stability. In the end, problems in analyte stability will surely attenuate effect size. (66)

Investigators can only control for, and properly interpret, the effect of stability if they understand what happens to the analyte of interest when it is in the freezer for any given length of storage. (52, 66, 75, 79, 84, 85) The first question is the shape of the degradation pattern of the analyte over time. (75, 84) Does the decline happen in a linear downward slope? Does it have an upward slope? Does it incrementally change? Investigators must consider if storage time affects the assay's precision to avoid reducing statistical power, as noted above, or introducing bias. (7, 9, 12, 75, 84, 87) For example, if the analyte changes occur differentially in relation to subject characteristics in a non-random fashion, bias can be introduced. (66, 84) For instance, some vitamins decline differently depending on the original concentration quartile, and because of the action of other substances in serum (e.g. EDTA, lipid peroxides, or antioxidants). (9, 24, 58, 67) Of course, analytic measures that start in the bottom quartile may degrade away entirely,

but evidence exists that for some analytes, starting in the upper quartile may result in faster initial concentration decline—in either case this can be controlled for but only if it is understood and accounted for in the analytic plan. (66, 81, 87)

Study methodology and protocols may create an environment where stability is the critical issue in the analytic model or may introduce bias into measurement results. (7, 9, 12, 22, 85) First, cohorts often take several years to be assembled. Biological samples must then be stored for varying lengths of time. Matching on length of storage must be undertaken to avoid influencing results. (75, 77, 79) Nested case-control studies are methodologically well-suited to study biomarkers, but the resulting long storage time requires an understanding of, and planning for, the effect of stability on analyte concentration. (75, 77, 79, 87) Care must be taken to ensure that all samples are initially collected, processed, stored, and analyzed with the strictest adherence to a uniform protocol that is identical for cases and controls. (15, 87) Multi-center trials often have demographic characteristics that vary by site requiring investigators to control for site to avoid introducing non-random between subject variations. (63, 75, 87) For instance, in the European Prospective Investigation into Cancer and Nutrition (EPIC) recruitment began in Oxford which had a higher SES and more vegetarians. (41, 73) As a result, both storage time and a lifestyle risk factor were systematically different by site and confounded with each other. (73)

Freeze-thaw cycles of the specimens must be considered as both a biological and methodological issue. (3, 51, 62, 74, 75, 77) For many analytes it appears to not matter how many cycles they incur, but for some, these cycles set in motion biological processes (introduction of moisture, oxidation, etc.) that impact upon ultimate concentration. (3, 12, 84) The number of freeze- thaw cycles may need to be accounted for by matching samples on the

number of cycles. (72, 84) This problem can also be minimized by storing specimens in multiple small aliquots. (84, 87)

Ocke and colleagues demonstrated, in a small simulation of carotenoid and niacin levels, that random between-subject differences in declining concentration tended to bias the effect measurement toward the null. (66) They concluded that the simulation was too small to make any precise estimation of effects, but rather demonstrated that stability must be understood "to be able to correct the epidemiological measures of effect for attenuation". (66)

Beyond the scientific reasons to understand stability, the cost and carbon footprint of long term ultra-low temperature storage should motivate exploring the highest storage temperature required to produce viable specimens, because the lower the temperature the higher the energy consumption and the carbon footprint. (20) The cost of -20° C storage is about one third of the cost of -80° C. (20) Electrically powered mechanical refrigeration looms large on the horizon for long term bio-specimen storage as the world adopts carbon taxes and other measures to reduce global warming. The cost of ultra-low temperature storage can only increase making it critical for specimens to be stored at the scientifically required temperature to produce a reliable assay of a given analyte. (20, 39)

# CHAPTER 1: METHODS

## A. Search Criteria

The National Library of Medicine's website, PUBMED, was systematically searched for articles relevant to the stability of stored blood that were published in peer reviewed journals from 1970 – 2013. Search strategies were developed using combinations of the following medical subject heading (MeSH) keywords: *nested case control and cohort samples, biostorage, temperature, biobanking, specimen storage, storage conditions, degradation, stability, and validity. T*he author conducted a separate review of each analyte of interest with the MeSH words *stability of stored "analyte" and validity of stored "analyte"* to ensure that the storage validity literature of specific analytes of interest was completely captured. For example, the fat soluble vitamin A compound retinol was searched for individually with the following phrases: "stability of stored retinol" and "validity of stored retinol". A total of 436 potential titles were identified via the two search strategies and articles were considered relevant if the title included some combination of an analyte, temperature, stability, validity, or stored samples. A review of the relevant titles' abstracts and methods resulted in 109 full articles retrieved for review.

The National Collaborative Perinatal Project (NCPP) bibliography and the International Society of Biological and Environmental Repositories (ISBER) website were cross referenced to establish that all relevant articles were retrieved. The NCPP bibliography contributed 3 additional articles and the ISBER bibliography posted in February of 2009 lead to the discovery of 2 additional articles. The citations of all included articles were systematically searched and an additional 9 articles were added to the review database. Articles were excluded if the paper was

not available in English (n=2). This review does not include unpublished data or scientific conference abstracts or reports (n=3).

B. Inclusion Criteria

This search strategy resulted in 68 peer reviewed articles included for the final review. To be included studies had to store specimens for a minimum of 6 weeks (42 days) and delineate storage and analyte measurement methods. Initially the inclusion criteria only included true validation studies, but the author quickly realized that a significant number of papers would be excluded if all studies were required to have baseline measurements. The inclusion criteria were expanded to catalog less robust representations of validity, and the author developed a system, modeled after US Preventive Services Task Force recommendation ranking, to characterize articles by the rank order level of evidence that they contributed to the science of stored specimen stability.

C. Literature Matrix

The gold standard for specimen stability validation requires storing blood at a predetermined temperature, measuring the quantity of a given analyte at baseline (time of collection), and continuing to measure the quantity of the analyte at specified time intervals with the goal of documenting the effect of storage over time. Validation studies that estimated baseline analyte values with a robust statistical process were still considered to be a true validation study or *level A* literature (n= 28). (Appendix table 1) Studies that compared stored specimens' values to specimens from the same archive that were tested after baseline were labeled *level B* evidence (n = 2). Studies that compared results to fresh specimens' measurements were designated *level C* evidence (n = 5). Finally, *Level D* (n = 9) evidence was defined as comparing study measurements to other published measurements of the analytes of interest

stored at a similar temperature and ideally for a similar number of years. The literature rank order scheme is summarized in appendix table 1.

Storage temperature validity is ultimately driven by the analyte of interest, thus articles were further organized by the analyte being evaluated for storage stability. Nutritional markers and steroid hormones are the primary focus of this review. Literature review summary tables were created that organized the articles by analyte, by storage temperature, and finally by the level of evidence that the article contributed. (Appendix tables 2 - 9)

# CHPATER 2: NUTRITIONAL RESULTS

## A. Nutritional Biomarkers Background

The epidemiological literature is full of explorations of the role of nutritional markers in everything from pregnancy related conditions to cancer, and heart disease. One can easily find multiple large cohorts that have stored biological specimens for the purpose of supporting cohort and case-control studies that have or will evaluate the effect of nutritional markers on disease outcomes. One notable example is the European Prospective Investigation into Cancer and Nutrition (EPIC) which is poised to answer a multitude of questions with approximately 520,000 participants' health and lifestyle information and a biological repository. (73) Biological samples from many thousands of participants have been at the center of the nutritional hypotheses studied within The Japan Collaborative Cohort (JACC) Study for the Evaluation of Cancer Risk and The National Health and Nutrition Examination Survey (NHANES). (63, 70) The largest perinatal repository is the Finnish Maternity Cohort (FMC) with 1.3 million first trimester serum samples. (33) Add to these larger cohorts the numerous smaller projects with biological specimens and the significant number of stored specimens residing in freezers all over the world can be appreciated.

Despite all of this activity knowledge is lacking regarding the stability of stored nutritional analytes. (9, 35, 67) Nutritional investigators have complained that they lack a validated map describing how the nutritional markers behave when stored at a particular temperature for a given period of time. (63, 66) Many investigators attempt to describe the stability of stored analytes while also investigating the analytes' role in disease. (16, 38) The cohort and case-control literature is full of studies that attempt to validate measurements they

produce without stored sample validation being a hypothesis they intended to study. (37, 38, 40) Commonly these papers will assure readers that their samples are valid by referencing unpublished results from their lab, by drawing some fresh specimens from staff, comparing to the results of similar studies, or by simply arguing that all samples experienced the same degradation. (2, 18) The resulting body of evidence relies more heavily on expert opinion or less robust determinations of stability than on the results of studies designed to validate the degradation of a given analyte at a given temperature.

This review included 25 nutritional analyte articles that had stored specimen stability as a study hypothesis, and spanned the breadth of quality from validation studies with baseline measurements (level A) to validation studies that compared results to published results (level D). The final search strategy for this paper discovered 18 papers that did not have nutritional biomarker storage validation as a hypothesis and they were eliminated from the review.

Reviewed articles are organized first by type of nutritional marker and then by the standard vitamin classification scheme and subgroups: A) fat soluble vitamins 1) vitamin A 2) vitamin D 3) vitamin E and B) water soluble vitamins 1) vitamin C 2) vitamin B1, B2, B3, B6, B9, and B12. Next the papers were organized around storage temperature (-80/-70° C, -25/-20° C, or both), and finally by the level of evidence (level A, B, C, or D). A summary table of papers included in each nutritional section is presented in the appendix (table 2 – 9) -- results are presented as annual percent change unless noted otherwise.

B. Fat Soluble Literature

1. Fat Soluble Literature: Retinol and Vitamin A

Retinol is the most commonly studied vitamin A compound with a total of 14 studies, 3 of which contributed level A evidence at -80 storage. (Appendix table 2) Level A evidence

or true validation studies require the use of baseline analyte measurements—two studies had baseline measurements and one study estimated baseline with a regression line created from measurements at three time points (15.5, 27.5, and 51.5 months). (17, 37) The reported percent change for retinol calculated from a regression line was -0.49% annually (17). Comparison to actual baseline measurements resulted in an annual percent change of -0.5 % after 9 years of storage (37) and an annual change of 1.5% reported after approximately 28 months. (19)

Retinol stability was validated in two studies that met the criteria for level C evidence (Appendix table 2) utilizing approximately 200 specimens stored for 10 and 8 years at -80° C. (86, 65) Willett and colleagues used fresh samples and reported an annual change of 0.76% after the 10 years of storage (86) and Nomura and colleagues used stored volunteers' samples and reported a -0.5% annual change after 8 years (65).

Few studies do a direct comparison of more than one storage temperature. This review found three studies of level A quality that compared retinol stored at -80° C and -20° C after 5 and 15 months. (Appendix table 2) (19, 11, 24) At both temperatures and time points the mean retinol value was nearly identical—0.52 mg/L at 5 months versus 0.53 mg/L at 15 months for -20° C and 0.55 mg/L at 5 months versus 0.58 mg/L at 15 months for -80° C. (19) A study from a large storage bank in Norway that compared -80° C and -25 ° C for 1 year concluded that the colder temperature was "statistically more stable". (11) A comparison of dried blood spots and frozen sera at the two temperatures was conducted for 6 weeks and the two storage mediums had "highly correlated" measurement values. (24) Both temperatures were included in an often cited review paper that contributes level C evidence. For their review Comstock and colleagues used published results to calculate mean values weighted by

the number of samples included in the studies.(18) Results from studies of fresh sera were compared to a weighted mean from studies of sera stored at -80° C and -20° C for 6 months to 13 plus years. (18) The weighted mean for retinol in fresh sera was reported as 780 mcg/L. (18) Retinol stored at -80° C had a reported weighted mean for 6 months to 3 years of storage of 566 mcg/L, 4 to 7 years of storage of 687 mcg/L, 8 to 12 years of storage of 596 mcg/L, and 13 plus years of storage of 616 mcg/L. (18) The respective percent changes are -27%, -12%, -25%, and -21%. (18) Retinol stored at -20° C had a reported mean for three time points—549 mcg/L at 4 to 7 years of storage, 581 mcg/L at 8 to 12 years of storage, and 635 at 13 plus years of storage. (18) The respective percent changes are -30%, -25%, and -18%. (18)

Four level A validation studies and one level C study evaluated the stability of retinol stored at -20° C for 1 to 16 years. (Appendix table 2) The endpoints for the level A studies were relatively short with reporting after one year of storage. Three studies reported retinol to be stable with annual percent changes of -1.9% (82), -0.8% (74), and -1.2% (71). Barreto-Lins and colleagues reported that the "exponential constant of destruction was O". (4) The level C study stored specimens for 16 years and compared the stored value to fresh samples stating that "the retinol was stable". (42)

Four studies that we found reported a total vitamin A value instead of the more common protocol of reporting individual vitamin A compounds. (Appendix table 2) Level A evidence was produced from sera obtained from NHANES II samples that were stored for 5 to 8 years and were reported to have a stable coefficient of correlation. (21) The vitamin A mean values had an annual percent change of -0.4% after 8 years. (21) A significantly higher annual decline of 32% from the baseline vitamin A values obtained from 55 samples was found with

2 years of storage in the other included study. (66) Storage for one year was completed by two level A studies that found vitamin A to be stable. Stability was represented in an annual percent change of 0.7% (36) and in a line plotted that had an r-square of 0.9128 with time 0 and time one year measurements. (27)

2. Fat Soluble Literature: Total Carotenoids, Alpha-Carotene, and Beta-Carotene

Three studies were found that reported on total carotenoids at both -80 and -20 and spanned the evidence range of A to C. (Appendix table 3) The level A study reported "no significant change" in the mean carotenoid value without publishing the measured values after 1 year of -80 storage. (59) The other -80 paper met level C quality criteria and had percent change ranges for the 10 years of storage duration of 0.3% to 4.6%. (86) The Mathews-Roth and Stampfer paper reported on an experiment of carotenoid storage at -20 and noted rather inconsistent annual percent changes-- at 6 months the carotenoid annual percent change was -30% and at 10 years it was -9.7%. (60)

Alpha-carotene was evaluated in three level A papers that also included retinol. (Appendix table 3) Alpha-carotene stored had an annual percent change of 0.75% after 4 years at -80° C (18), and -1.6% after 9 years (37), and 0.84% after 28 months (20). The validation study published by Craft et al. that included storage at both -80° C and -20° C reported that after 15 months at -80° C the α-carotene was stable, but after 15 months at -20° C α-carotene had suffered a "statistically significant decline". (20) The -20° C stored samples had the same mean as the -80° C samples at 5 months, but the -80° C plasma at 15 months had a mean of 1.2 mg/L whereas the -20 plasma had a mean of 0.7 mg/L. (20) The final α-carotene paper stored sera at -20 for one year and reported an annual percent change of -7.5%. (82)

Beta-carotene was included in two of the previously reviewed level A papers at -80° C storage and was reported to show little to no change in values. (Appendix table 3) The annual percent changes were 0.32% after 4 years of storage (17) and 1.5% after 28 months of storage (19). Two additional level C papers found inconsistent β-carotene values—after 13 years of storage there is a report of an annual decrease of -0.87% (18) and after 8 years of storage an annual increase of 7.3% (65). Finally, β-carotene was included in a comparison between colder and warmer temperatures (-80° C and -20° C) and like α-carotene had "a statistically significant decline" in mean value at the warmer temperature (1.49 mg/L versus 0.8 mg/L). (19)

We found three β-carotene studies that evaluated -20° C storage with three contributing level A evidence and one contributing level C. (Appendix table 3) All three level A studies reported significant declines in mean values of sera stored at -20° C. (66, 76, 82) After two years Ocke and colleagues and Thurnham and colleagues report annual declines of 43% and 22.4%, respectively. (66, 84) Smith and colleagues report a statistically significant mean decrease of 50 mcg/L per year. (76) The level C review paper, discussed previously with other analytes, reported an annual percent change of -4.7% after 13 plus years of storage. (18)

3. Fat Soluble Literature: Cryptoxanthin, Lutein, and Lycopene

The remaining vitamin A compounds are generally evaluated together because of their similar chromatographic spectrum. (80, 82) Table four (appendix) summarizes the three studies that evaluate cryptoxanthin, lutein, and lycopene individually. This review includes one level A study at -80 that reported all three as having an annual percent change less than 2.2% after 9 years of storage. (37) The Comstock and colleagues level A paper reported an annual percent change for each of three compounds after 4 years of -80 storage—

cryptoxanthin 1.03%, lutein 0.18%, and lycopene 0.03%. (18) One level A study evaluated β-cryptoxanthin and lycopene at -20° C storage for 1 year and reported mean decreases respectively of -26% and -21%. (82)

4. Fat Soluble Literature: Vitamin D

The storage validity of vitamin D was discussed in four level A papers. (Appendix table 5) In a study designed to determine temperature validity and how analytes respond to serum separator gel, Mathew and colleagues report that "there is no change in mean vitamin D level" when stored for 12 months at -80 without serum separator gel. (58) If sera is exposed to serum separator gel the mean increases by 37% in 12 months. (58) The Finnish Maternity Cohort was the source of samples that had been stored for 6 to 24 years at -25° and vitamin D was determined to be "stable". (1) A slightly warmer temperature (-20° C) was tested for a year and vitamin D was also found to be "minimally changed", (49) but the final study at -20° C found a somewhat variable percent change. (66) Ocke and colleagues found that at 12 months vitamin D had declined by 5.5%, at 24 months had declined by 10.9%, at 36 months was unchanged, and at 48 months had declined by 13.7%. (66)

5. Fat Soluble Literature: Vitamin E, Tocopherols, Alpha- tocopherols and Beta-tocopherols

The stability of Vitamin E as a complete compound was evaluated in four validation studies discovered by this reviewer. (Appendix table 6) All studies stored sera at -20° C from 6 weeks to 2 years. After 2 years of storage vitamin E declined annually by -0.46% (66), at one year had declined annually by -2.8%, (36) and at 6 weeks of storage (the minimal length of storage required to be included in this review) declined annually by -0.5%. (16) Again, Gunter and colleagues plotted the baseline and end of storage values on a line with vitamin A's values having an r-square of 0.6796 or moderate stability. (27)

Measurement of tocopherols was the more common way to examine vitamin E stability. (Appendix Table 6) Total tocopherols had an annual percent change of 1.5% reported by Craft and colleagues after 28 months at -80° C storage. (19) The Craft team also measured total tocopherol stored at -80° C and -20° C for 5 and 15 months with quite discrepant results. (19) The annual percent change for -80° C was 18% and for -20° C was -1.4% (19)

Alpha-tocopherol was measured in two reviewed level A studies and in two reviewed level C study at -80° C storage. (Appendix table 6) Comstock and colleagues again contribute level A evidence with 4 years of storage at -80° C and an annual percent change of -0.41%. (17) One other level A paper at -80° C storage reports an annual percent change of 0.5% after 9 years of storage. (37) Annual percent changes of -0.02% and -0.11% were reported by two level C articles after 10 and 13 years, respectively, of -80° C storage. (86, 18)

A higher storage temperature (-20° C) was investigated for α-tocopherol and ϒ-tocopherol. A level C paper was the final paper for α-tocopherol and reported a -2.4% annual change in 13 plus years. (18) Comstock and colleagues' level A paper included ϒ-tocopherol and found an annual percent change of -0.92% after 4 years of storage. (17)

C. Water Soluble Literature

1. Water Soluble Literature: Vitamin C

Five validation studies with baseline measurements have explored the stability of vitamin C, commonly measured as ascorbic acid (AA). (Appendix table 7) Samples that are intended to determine ascorbic acid levels are generally stabilized with metaphosphoric acid (MPA) or with dithiothreitol (DTT). (55) Three of the five studies investigated the stability of ascorbic acid and evaluated the effect of stabilization with MPA or DTT on AA levels. (Appendix table 7) The first validation study summarized in table 7 stabilized samples with

MPA and after 4 years of -80 storage a -0.66% annual change was reported. (17) Margolis and Davis evaluated DTT's effect on mean values and two different storage mechanisms; freeze dried frozen storage and standard frozen storage. (53) The samples that were freeze dried had a -1.6% annualized change after 80 weeks and the samples that underwent standard freezing had no change reported after 57 weeks. (53). One final level A study that stored samples for 2 years found an annual percent change of -6.8%. (43)

One validation study compared MPA and DTT and evaluated two different ways of measuring vitamin C—ascorbic acid or total ascorbic acid (AA + dehydroascorbic acid). (Appendix table 7) After 6 years of -80 storage they report that TAA measurements were quite stable when the sample was stabilized with DTT—0.1% and 0.2% annual change in two stored sample lots. (54) TAA with MPA stabilization was also stable with -1.1% and -0.9% annual change reported. (54) However, simple measurement of AA was not as stable when MPA alone was used and the percent change declined to -4.1% and -7% annual change. (54)

Investigators with the EPIC cohort sought to determine what would happen to AA if the sera were not stabilized because, they argue, most sera collected by epidemiologic studies would not be stabilized before storage. (41) They measured AA in sera that had been stored at -196 for 7 to 11 years and report moderate correlation between the baseline values and the stored values. The annualized change was -3.3% to -2.1% in 7 to 11 years. (41)

2. Water Soluble Literature: B Vitamins

The B vitamins are not widely studied and most of the interest is focused on folate. One level A validation study found by this reviewer examined folate at three storage temperatures (-20° C, -80° C, and -196° C) for 12 months. (Appendix table 8) Folate was not stable at -20° C declining approximately 40% by 4 months, but had an unchanged rank order during the 12

months of storage. (40) At -70° C or -196° C the authors report a stable mean at 12 months and actually report no change “except expected measurement variation” in mean values. (40) The authors conclude that there is no need for -196 storage when folate is the analyte of interest. (40)

Hustad and colleagues used samples from the Janus Serum Bank in Norway that had been stored at -25° C for 29 years to explore degradation kinetics. (Appendix table 8) Folate declined approximately 2.9% annually and had a mean decline of approximately 50% at 29 years. (35) Another level A study that used the Janus Bank samples reported a similar annual decline of -2.9% to -3.4% when folate is measured as 5-methyltetrahydrofolate (5mTHF), however if folate’s degradation products are also captured and measured as p-amniobenzoylglutamate (pABG) then the annual percent change is -0.7% after measured at 29 years. (29) Ocke and colleagues analyzed folate as 5mTHF and as with other analytes report a higher percent change of -5.6% after 4 years at -20° C. (66) One final level A study included stored spiked specimens at -20° C for a year and reported “no degradation” of folate or a 1.2% annual change during storage. (36)

Vitamin B1 (thiamine), B2 (riboflavin), and B6 (pyridoxine) each had two level A validation studies. B1 (thiamine) decreased -0.8% (66) and -1.8% (36) after 4 years and 1 year, respectively, of -20° C storage. (66, 35) Two of the previously cited papers included B2 (riboflavin) and had somewhat discordant findings. (66, 35) First the Janus Serum Bank contributed evidence of a 2.6% annual percent change after 29 years of -20° C storage (35) whereas the other level A paper reported a -0.9% annual change after 4 years of -20° C storage. (66) Vitamin B6 (pyridoxine) findings were also somewhat inconsistent. (35) After 29 years of -25 exposures B6 declined by -0.86% annually (35), but after 4 years of -20 exposure B6 had declined by -3% annually. (66)

Four papers also probed the stability of vitamin B12 (cyanocoblamin), and again Jansen and colleagues compared stability at -196° C, -80° C ,and -20°C over a year and reported it to be "stable" at all temperatures. (40) The Janus Serum Bank provides stability evidence with measurements of vitamin B12 increasing 0.35% annually at -25° C (35) and increasing 1.9% annually at -20° C. (66). Finally, spiked samples utilized by Ihara and colleagues produced a -0.09% annual change at -20° C. (36)

D. Nutritional Biomarkers Discussion

Overall, the fat soluble vitamin A compounds appear reasonably stable. Retinol is the most commonly studied vitamin A analyte with a total of fifteen studies and has the most robust collection of validation studies. Retinol's annual percent change was reported as -0.5% to 1.5% for -80° C storage (37, 17) and -1.9% to 1.2% for -20° C storage. (82, 71) (Appendix table 2) Two validation studies reported retinol as stable when directly comparing both temperatures while one comparison study reported -80° C as "slightly more stable". (11, 16, 17, 18)) Stability is reported for total vitamin A in four level A papers, however Ocke and colleagues reported an annual percent change of 32%. (21, 28, 36, 66) A thorough review of the Ocke study did not reveal why this annual percent change was an outlier. (66) Given the body of reviewed literature it seems reasonable to use -20° C storage if retinol or vitamin A is of interest, especially if storage is limited to a maximum of 10 years.

Total carotenoids and α-carotene each have 3 or 4 studies covering -80° C and -20° C which limits the conclusions that can be drawn about these analytes. (Appendix table 3) That said, this review would cautiously support -80° C storage for carotenoids and α-carotene. (16, 17, 18, 35, 58, 60, 82) Beta-carotene, included in a total of 9 studies, appears to require -80° C for stable maintenance. (16, 17, 18, 65, 66, 74, 82) Limited data precludes meaningful conclusions

about the remaining vitamin A compounds (cryptoxanthin, lutein, and lycopene). (Appendix table 4)

Vitamin D has three level A studies at both -80° C and -20° C that report stability. (Appendix table 5) (1, 49, 58, 66) Again the outlier is the Ocke et al. study and it is difficult to understand why this study is consistently an outlier. (66) This analyte is more a steroid hormone than a vitamin and may be determined to have stability patterns more similar to the other steroid hormones, but it requires more study to create a body of evidence from which to establish a robust storage protocol. (Appendix table 5)

Vitamin E and compounds appear to have, what one investigator called, “intermediate stability”. (17) Total vitamin E was reported in 4 level A studies to have a -0.5% to -2.8% annual change at -20° C and total tocopherol was reported in 1 level A study to have a -0.009% annual change at -80° C. (16, 27, 36, 66) Alpha-tocopherol with 4 studies at -80° C and one at -20° C is reported to have minimal annual percent changes of -0.02% to 0.5%. (17, 18, 37, 86) A higher storage temperature may be acceptable for these vitamin E analytes, but further investigation is required before full support of this conclusion can be given.

The water soluble vitamins were unique because all the studies included were level A studies. (Appendix table 7) Ascorbic acid (vitamin C) requires acidification before storage and stability varies by the type of acidification used. (53, 54, 55, 56) The most stable protocol reviewed uses TAA and either DTT or MPA and results in very minimal annual percent changes (0.1% - -0.9%). (17, 53, 54) However, if MPA is used alone or if vitamin C is not acidified then the annual percent change is reported as high as 7%. (41, 43)

Folate and B12 were the most commonly studied B vitamins and the majority of studies used -20° C or -25° C. (Appendix table 8) Folate had reports of an annual percent change as high

as -5% and B12 had reports of annual percent changes of -0.09% - 1.9%. (35, 40, 29, 36, 66) Vitamin B1, B2, and B6 were also included with only two studies each at -20° C and had percent changes of -3 to 2.6, (35, 40, 66) B vitamins in general, folate especially, are significantly influenced by the analytic technique that is used to recover them. (35, 38) Hannisdal and colleagues found that folate measured as 5-methyl-tetrahydrofolate had a 3.2% annual change but when measured as p-amino-benzoylglutamate had a 0.7% annual change. (29)

The storage of vitamin analytes, as illustrated by the studies collected for this review, is a complicated endeavor requiring careful consideration of multiple ancillary lifestyle issues when developing an analysis plan that accurately assesses storage stability. (37, 38) Otherwise, studies are less likely to have internal and external validity. (32, 46) For instance, participants' dietary intake patterns can influence the measured levels of vitamin analytes. (69, 76) Ozasa and colleagues also have documented how diet and particularly the diets of ethnic groups as well as gender differences can also influence measured levels of stored samples. (69) Recent dietary intake, a fasting or non-fasting state, smoking and alcohol, total cholesterol, and even season differences of vitamin levels can influence the measured value of stored vitamins. (3, 9, 12, 25, 38, 43, 59, 69) Level A studies included in this review are likely to have pre-analytic bias because of such issues. Stability studies (level B-D) that do not have a baseline analyte measurement but depend on the results of another study or a collection of fresh samples to establish a benchmark of stability can be significantly influenced by the characteristics of the comparison group or study that is chosen.

Collection and processing procedures as well as laboratory analysis variability further conspire to influence the results and thus generalizability of stability studies. (27, 33, 42, 47, 86) The water soluble vitamins appear to be the most susceptible to inter-lab variability, in particular

Ihara, et al. and Jansen, et al. are influenced by lab variability. (36, 40, 56)The anticoagulant present in the blood collection tube, the time from freezer to specimen analysis, specimen exposure to air, light, and temperature in the lab have all been documented to effect the measurements of a given vitamin. (9, 79, 80, 86 Vitamin A levels are particularly vulnerable to oxidation and Comstock and colleagues could have been significantly biased due to oxidation. (17, 18) The number of freeze-thaw cycles before analysis can influence vitamin A compounds' results, but is not an issue for vitamin E. (27) Unfortunately, few of the included studies discuss in their methods the precautions that they take to protect samples from degradation before and during storage or when withdrawn for analysis in the laboratory. (4, 66, 73) Finally, the laboratory analytic technique used can effect results limiting the comparisons between and the conclusions drawn from the studies included in this review. (66, 86)

The primary objective of this review was to attempt to answer what storage temperature provides stability for vitamins and pro-vitamins? We assembled an original view of the available evidence—robustness of the data by storage temperature with stability trends noted or revealed. Additional research is urgently needed on the long term stability of vitamins and pro-vitamins at different temperatures over varying lengths of time. We will not know with confidence how vitamins are associated with disease processes or outcomes unless we first understand the degradation curve of a given vitamin of interest in the different quartiles at baseline and how the quartile measurements respond to varying lengths of time in storage. In short, we need to answer not only what temperature best preserves each vitamin, but also how does each vitamin decay? Answers to these questions are required to begin the process of establishing temperature storage protocols that allow investigators to be assured that their results are valid.

# CHAPTER 3: STEROID HORMONE RESULTS

## A. Steroid Hormones Background

PubMed has well over 2000 articles of case control investigations of steroid hormones' role in CVD and cancer. Yet 31 articles are retrieved if PubMed is searched for "stability of stored cortisol", cortisol being one of the more commonly implicated hormones. Little is known about the stability of stored steroid hormones despite significant activity and interest in their role in not only CVD and cancer but also mental health, obesity, diabetes, and cognitive development. (32, 44, 48, 77)

The PubMed search strategy outlined in the methods section retrieved a total of 23 papers with abstracts that suggested methods to investigate the stability of stored steroid hormones. One paper was found by manually searching the ISBER bibliography and two papers were found by reviewing the bibliographies of all papers. Unfortunately, only 12 of the retrieved papers met the inclusion criteria and are included in summary table nine (Appendix). The others were excluded because they did not store specimens for a minimum of 6 weeks or the methods did not include validation of a steroid hormone's storage temperature.

The steroid hormone results and summary table (appendix table 9) are organized first by analyte—estradiol, estriol, estrone, progesterone, prolactin, total testosterone, free testosterone, cortisol, androstenedione, sex hormone binding globulin (SHBG), and corticosteroid binding globulin (CBG). Each analyte is then organized by storage temperature, by the A to D level of evidence matrix, and finally by years of storage. When possible an annual percent change is presented otherwise the article's conclusion is directly quoted.

## B. Steroid Hormone Literature: Estradiol

One of the most often cited studies of steroid sex hormone stability is an Italian study that included both pre and postmenopausal women (n=16) whose serum and plasma were tested at baseline and stored at -80° C for 3 years. (8) (Appendix table 9) A -0.14% annual change was reported for premenopausal serum and 0.23% annual change was reported for postmenopausal serum--supporting the conclusion that estradiol is stable in serum.(8) Estradiol did not fair so well in plasma--the premenopausal and postmenopausal plasma percent changes were significantly higher at -5.6% and -6.8% respectively. (8) Cauley and colleagues report in a level C paper that estradiol was "stable" after 10 years of -80° C storage. (13) Level D criteria is met by two studies that report "unchanged" or "stable" after 15 years of -80° C storage. (5, 26) Higher temperature (-20°C) storage also is reported to adequately preserve estradiol in three level D studies. (32, 33, 88) The Finnish Maternity Cohort supplied serum from 154 participants and reported estradiol "to increase with storage time, but remain in the clinically relevant range" after -25° C storage for 29 years. (33) Henderson and colleagues reported "stability" of estradiol in specimens that had been stored at -20°C for 21 years when compared to samples stored for 6 years. (32) Finally, estradiol "stability" was supported by a level D study that utilized 814 NCPP participants' samples stored for 40 years at -20° C. (88)

C. Steroid Hormone: Estriol and Estrone

Estriol is reported to be "stable" at each of the storage temperatures in two level D papers. (Appendix Table 9) Again the work of Barrett-Connor and colleagues supports the stability of estiol when stored for 15 years at -80° C (5) and the NCPP sera supported the stability of estriol after 40 years at -20° C storage. (88) Estrone was reported to have unchanged means by a level C paper at -80° C for 10 years and by a level D paper for 14 years. (13, 26)

D. Steroid Hormone Literature: Progesterone and Prolactin

Progesterone and prolactin were investigated by two studies that met the inclusion criteria for this review. (Appendix table 9) The progesterone results are somewhat contradictory. (Appendix table 9) Progesterone's premenopausal annual percent change in serum was -14.4% and in plasma was -13.1% after 3 years of storage at -80° C. (8) Bolelli and colleagues also report that the rank order of progesterone was maintained throughout storage rendering the specimens valid for epidemiologic investigations. (8) Progesterone was reported to "decrease with storage time, but remain in the clinically relevant range" after 2-22 years at -25° C in a level D study. (33) Prolactin, only investigated in one paper, had an annual percent change of -1.4% and 1.4% in pre and postmenopausal serum and premenopausal plasma. (8) Postmenopausal plasma had a 2.3% annual change. (8)

E. Steroid Hormone Literature: Total and Free Testosterone

Total Testosterone measurements were contributed by Borelli and colleagues' who assayed both pre and postmenopausal women's serum and plasma after 3 years of -80° C storage. (8) (Appendix table 9) Premenopausal serum and plasma and postmenopausal plasma had an annual percent change of roughly 3.5% and postmenopausal serum had an annual percent change of -1.5%. (8) "Unchanged" or "stable" mean measurements were reported by three studies that compared samples stored at -80°C to fresh samples (level C) or to published results (level D). (5, 13, 26) A previously reviewed level B paper also reports "stable" total testosterone measurements after 21 years of storage at -20° C.(32) The NCPP, -20° C storage for 40 years, contributed total testosterone data in three studies. Stroud and colleagues reported a 0.09% annual change in samples drawn during the third trimester and a -0.08% annual change drawn the day of delivery when compared to published means from fresh samples. (77) The final paper reporting total testosterone stability in the NCPP samples did not include actual data. (88)

Free testosterone had a 7.8% annual change in the only level A validation study. (8) However, Cauley and colleagues found similar means when comparing the measurements of stored free testosterone to published results. (13)

F. Steroid Hormone Results: Cortisol

Cortisol was included in the study of the NCPP samples and those drawn in the third trimester had an annual percent change reported as –0.044%. (7) Cortisol drawn on the day of delivery had an annual percent change of -0.87%. (77)

G. Steroid Hormone Results: Androstenedione

Androstenedione, the precursor to male and female sex hormones, was measured in two -80° C studies for 10 and 14 years. (Appendix table 9) A level C paper reported that androstenedione was "unchanged" when compared to fresh samples after 10 years of -80° C storage. (13) Stability was also reported with four additional years of storage at -80° C and comparison to the author's unpublished results. (26)

H. Steroid Hormone Binding Globulins: Sex Hormone Binding Globulin and Corticosteroid Binding Globulin

Sex Hormone Binding Globulin (SHBG) has no level A validation studies. (Appendix table 9) SHBG is stable according to two level D studies at -80° C storage for 14 and 15 years that have been discussed with other analytes. (13, 26) The -20° C papers go from level B to level D literature and range from 16 to 40 years of storage. The level B paper reported stable measurements for SHBG when compared to samples in their archive measured over a 6 year period (32) and one level C paper also reported stability. (48) Stroud and colleagues are the only investigators to actually report the results they were comparing to allowing annual percent change to be calculated. Samples collected during the third trimester and on the day of delivery had an annual percent change respectively of -0.44% and -0.94%. (77) Corticosteroid binding

globulin (CBG) had a 0.43% annual change after 16 years of -20° C storage when compared to both fresh samples and published reports. (48) Finally, 40 years of -20° C storage resulted in a 0.54% annual change for third trimester samples and a 0.32% annual change for day of delivery samples. (77)

I. Steroid Hormones Discussion

Expert opinion abounds regarding the stability of steroid hormones—it has even been said that they are “the most stable of compounds”. (33) This expert opinion is possibly bolstered by the fact that steroid hormones appear to be unaffected by multiple (as many as 10) freeze thaw cycles. (51,72) Overall there is evidence that they are stable, however, there is only one true validation study by Bolelli and colleagues that includes estradiol, progesterone, prolactin, total and free testosterone. (8) The remaining studies rely on comparison to fresh samples or published results and generally do not report the comparison means.

The assembled literature appears to support that serum estradiol, estriol, estrone, total testosterone, and cortisol are stable. (8, 13, 77) Prolactin endures modest storage changes, but with a roughly 1% annual change is considered acceptable by most authors for epidemiological studies. (8) The results for progesterone and free testosterone are variable with contradictory results reported making it difficult to draw a conclusion. (8, 33) However, progesterone and free testosterone rank orders are maintained with very minimal variance which is considered by some authors to be the critical issue for epidemiological studies. (8) Bolelli and colleagues conclude that progesterone and free testosterone can be rank ordered instead of studied by mean measurements at least when stored at -80°C for 3 years. (8)

Androstenedione is reported in two papers to be stable. (13, 26) SHBG has some variability of results with four studies reporting no difference, one reporting an insignificant

increase, and the only study (76) with actual means to compare reporting a moderate decrease. (8, 13, 32) CBG appears reasonably stable with annual percent change of approximately 0.4%. (48, 77)

The generalizability of the steroid hormone and binding globulin studies is questionable for multiple reasons. Primarily the included studies provide level C and D evidence or compared stored results to fresh samples or published results which is an inferior substitute for level A evidence with baseline measurements. All studies are potentially challenged by methods variability and the collected literature for this review was particularly challenged. First, the comparison results were often generated by a different assay method, the labs were operating with different processing methods, and the specimens were stored varying lengths of time. Extraction assay kits changed during the study period of the level A study for estradiol, total testosterone, and progesterone, creating the potential for significant lab variability issues. (8) Cortisol and testosterone vary throughout the day and testosterone is affected by nutritional status yet few of the studies collected and reported this information.

Storage containers can further influence the measurements of steroid hormones and binding globulins. (77) Air tight storage containers have been shown to be critical for steroid hormones to prevent evaporation of the storage medium and an increase in measured mean hormone values. (72, 83) Unfortunately, limited information was included in the methods of reviewed studies regarding the type of caps used for storage. Finally, these studies may further be confounded by steroid hormones affinity for the plastic tubes that are commonly used in ultra-low temperature storage. (8, 12) Bruning and colleagues reported that this was especially true for progesterone and androstenedione because they are less polar molecules and thus more attracted to the plastic. (12)

Many of the papers presented in table 9 (appendix) share only limited methods, but more importantly do not share the analytes' measured means rather simply share the conclusion that the analyte is stable or unchanged. This especially limits the conclusions that can be drawn about a -80° C or a -20° C storage temperature despite a number of less robust -20° C papers supporting stability. The case can be made that either the comparisons made in the level B – D papers are like comparing apples to oranges or given the limited methods and data that it is simply difficult to know the strength of the conclusion.

Additional level A studies would add certainty to the claim that the sex steroid hormones and the binding globulins are stable. Planning for those validation studies requires rigorous attention to collection and processing methods that ensure time of day and fasting status is recorded when the sample is collected, the aliquots are in appropriate air-tight tubes, and an assay is chosen that is generally in use and not likely to change from the time when baseline measurements are taken until the study is completed. (83) Once samples are in the freezer they need to be maintained at a stable temperature with constant monitoring and logging of any freeze-thaw cycles. (72) Validation of analytes needs to occur on specimens that have been frozen a similar length of time and if from pregnant participants they need to be matched with baseline specimens from the same gestational age. (87) Validation studies that attend to these details would allow the development of a robust steroid hormone storage protocol that allows investigators to use stored specimens with confidence to explore steroid hormones' and binding globulins' role in multiple disease states.

# CHAPTER 4: CONCLUSIONS

Many millions of biological aliquots currently reside in freezers all over the world and some for as long as 50 years. Several thousand papers have been written with findings provided by these samples, yet few analytes have clearly and robustly delineated storage protocols or degradation curves. Without these it is difficult to properly develop specimen collection and storage methods or to properly control for potential confounding when analyzing results. The cost of studies with stored samples could potentially be significantly reduced if it was understood which analytes require -80° C and which are conserved at -20° C. In short, conclusions are tenuous, at best, that are drawn from vitamin or steroid hormone specimens. Validation of storage temperatures is required for molecular epidemiologic studies to progress forward and make their mark on multiple disease processes.

# APPENDIX

| Table 1: Literature Matrix Summary | |
|---|---|
| Level A | True Validation Study—baseline measurements at the time of collection. |
| Level B | Comparisons made with the same biological archive that the current study is using, but the comparison measurements are made at a time point other than baseline or the current study. Briefly, same archive different time point. |
| Level C | Comparisons made with measurements on fresh samples. |
| Level D | Comparisons made with the results of published measurements. |
| Level E | Expert Opinion |

| Table 2: Vitamin A and Retinol | | | | | | |
|---|---|---|---|---|---|---|
| **Vitamin A & Retinol** | **Study** | **Evidence Level** | **Storage Time** | **Storage Temp** | **Comparison Source** | **Annual % Change** |
| Retinol | Comstock et al, 1995 (#) | **A** | 4 years (51.5 months) | -80° C | Estimated baseline with regression line | -0.49% |
| Retinol | Ito (JACC) 2003 | A | 9 years | -80° C | Baseline | -0.5% |
| Retinol | Craft | A | 28 months | -80° C | Baseline | 1.5% |
| Retinol | Willett | C | 10 years | -80° C | Fresh samples | 0.76% |
| Retinol | Nomura | B/C | 8 years | -80° C | Stored “Normal volunteers ‘” samples | -0.5% |
| Retinol | Craft | A | 15 months | -80° C vs. -20° C | Compared means at 5 and 15 months of storage | Report “no change” |
| Retinol | Brown | A | 10 years | -80° C & -25° C | Baseline | More stable at -80 |
| Retinol | Fujita | A | 6 weeks | -80° C & -20° C | Baseline | DBS correlate with sera levels |
| Retinol | Comstock 1993 | C | 13 years | -80° C & -20° C | Fresh | 616 mcg/L (calc.) |
| Retinol | Thurnham | A | 1 year | -20° C | Baseline | -1.9% |
| Retinol | Rider | A | 1 year | -20° C | Baseline | 0.8% |
| Retinol | Peng | A | 1 year | -20° C | Baseline | 1.2% |

| Table 2 (cont'd) | | | | | | |
|---|---|---|---|---|---|---|
| **Vitamin A & Retinol** | **Study** | **Evidence Level** | **Storage Time** | **Storage Temp** | **Comparison Source** | **Annual % Change** |
| Retinol | Barreto-Lins | A | 1 year | -20° C | Baseline | "stable CV (2.5% - 3.5%)" & "exponential constant of destruction = O" |
| Retinol | Kark | C | 16 years | -20° C | Fresh Samples | "Stable" |
| Vitamin A | Driskell 1985 | A | 8 years | -20° C | Baseline | -0.4% |
| Vitamin A | Ocke | A | 2 years | -20° C | Baseline | -32% |
| Vitamin A | Ihara | A | 1 year | -20° C | Baseline | 0.7% |
| Vitamin A | Gunter | A | 1 year | -20° C | Baseline | "Stable" r-square =0.9128 |

| Table 3: Carotenoids (Alpha and Beta Carotene) | | | | | | |
|---|---|---|---|---|---|---|
| **Carotenoids: Alpha & Beta Carotene** | **Study** | **Evidence Level** | **Storage Time** | **Storage Temp** | **Comparison Source** | **Annual % Change** |
| Carotenoids | Willett | D | 10 years | -80° C | Published results | -0.3% - -4.7% |
| Carotenoids | Mathews-Roth | D | 12 months | -80° C | | "Essentially unchanged" |
| Carotenoids | Mathews-Roth & Stampfer | C | 6 months & 10 years | -20° C | | -30% & -9.7% |
| α-Carotene | Comstock 1995 | A | 4 years (51.5 months) | -80° C | Estimated baseline with regression line | 0.75% |
| α-Carotene | Ito | A | 9 years | -80° C | Baseline | -1.6% |
| α-Carotene | Craft | A | 28 months | -80° C | Baseline | 0.84% |
| α-Carotene | Craft | A | 15 months | -80° C vs -20° C | Baseline | "Stat. significant decline" |
| α-Carotene | Thurnham | A | 1 year | -20° C | Baseline | -7.5% |
| β-Carotene | Comstock 1995 | A | 4 years (51.5 months) | -80° C | Estimated baseline with regression line | 0.32% |
| β-Carotene | Craft | A | 28 months | -80° C | Baseline | 1.5% |
| β-Carotene | Comstock 1993 (review) | **C** | 13 years | -80° C | Fresh samples | -0.87% |
| β-Carotene | Nomura | B/C | 8 years | -80° C | "Stored "Normal volunteers '" | 7.3% |

| Table 3 (cont'd) | | | | | | |
|---|---|---|---|---|---|---|
| **Carotenoids: Alpha & Beta Carotene** | **Study** | **Evidence Level** | **Storage Time** | **Storage Temp** | **Comparison Source** | **Annual % Change** |
| β-Carotene | Craft | A | 15 months | -80° C vs. -20° C | Baseline (values not given) | "Stat. significant decline" |
| β-Carotene | Ocke | A | 24 months | -20° C | Baseline | -43% |
| β-Carotene | Smith | A | 2.75 years | -20° C | | Means decreased by 50 mcg/l annually |
| β-Carotene | Thurnham | A | 1 year | -20° C | Baseline | -22.4% |
| β-Carotene | Comstock 1993 (review) | C | 13+ years | -20° C | Fresh samples | -4.7% |

| Table 4: Other Vitamin A Compounds (Cryptoxanthin, Lutein, and Lycopene) | | | | | | |
|---|---|---|---|---|---|---|
| **Other A: Cryptoxanthin, lutein, & lycopene** | **Study** | **Evidence Level** | **Storage Time** | **Storage Temp** | **Comparison Source** | **Annual % Change** |
| Cryptoxanthin, lutein, & lycopene | Ito | A | 9 years | -80° C | Baseline | <2.2% |
| Cryptoxanthin | Comstock 1993 | C | 4 years | -80° C | Fresh Results | 1.03% |
| Lutein | Comstock 1993 | C | 4 years | -80° C | Fresh Results | 0.18% |
| Lycopene | Comstock 1993 | C | 4 years | -80° C | Fresh Results | 0.03% |
| b-Cryptoxanthin | Thurnham | A | 1 year | -20° C | Baseline | -26% |
| Lycopene | Thurnham | A | 1 year | -20° C | Baseline | -21% |

| Table 5: Vitamin D | | | | | | |
|---|---|---|---|---|---|---|
| **Vitamin D** | **Study** | **Evidence Level** | **Storage Time** | **Storage Temp** | **Comparison Source** | **Annual % Change** |
| Vitamin D | Mathew | A | 12 months | -80° C | Baseline | No change |
| Vitamin D | Agborsangaya | A | 6 – 24 years | -25° C | Baseline | No change |
| Vitamin D | Lensmeyer | A | 12 months | -20°C | Baseline | No change |
| Vitamin D | Ocke | A | 4 years | -20° C | Baseline | -13% |

| Table 6: Vitamin E & Tocopherols (Alpha and Beta Tocopherols) | | | | | | |
|---|---|---|---|---|---|---|
| **Vitamin E & Compounds: a-tocopherol, b-tocopherol** | **Study** | **Evidence Level** | **Storage Time** | **Storage Temp** | **Comparison Source** | **Annual % Change checked** |
| Vitamin E | Ocke | A | 2 years | -20° C | Baseline | -0.46% |
| Vitamin E | Clevidence | A | 6 weeks | -20° C | Baseline | -0.5% |
| Vitamin E | Ihara | A | 1 year | -20° C | Baseline | -2.8% |
| Vitamin E | Gunter | A | 1 year | -20° C | Baseline | "Moderately stable" r-squared = 0.6796 |
| Tocopherols | Craft | A | 28 months | -80° C | Baseline | 1.5% |
| α-tocopherol | Comstock 1995 | A | 4 years | -80° C | Estimated baseline with regression line | -0.41% |
| α-tocopherol | Ito | A | 9 years | -80° C | Baseline | 0.5% |
| α-tocopherol | Willett | C | 10 years | -80° C | Fresh samples | -0.02% |
| α-Tocopherols | Comstock 1993 | C | 13 years | -80° C | Fresh Samples | -0.11% |
| α-Tocopherols | Comstock 1993 | C | 13 years | -20° C | Fresh Samples | -2.4% |
| ϒ-tocopherol | Comstock 1995 | A | 4 years | -80° C | Estimated baseline with regression line | -0.92% |

| Table 7: Ascorbic Acid | | | | | | |
|---|---|---|---|---|---|---|
| **Analyte** | **Study** | **Evidence Level** | **Storage Time** | **Storage Temp** | **Comparison Value** | **Annual % Change** |
| Ascorbic Acid | Comstock et al, 1995 | A | 4 years (51.5 months) | -80° C | Baseline estimated regression line | -0.66 % |
| Ascorbic Acid | Margolis & Davis 1988 | A | 80 weeks | -80° C | Baseline | -1.6% |
| Ascorbic Acid (frozen) | Margolis & Davis 1988 | A | 57 weeks | -80° C | Baseline | No change |
| Ascorbic Acid | Karlsen | A | 2 years | -80° C | Baseline | -6.8% |
| Ascorbic Acid (TAA , DTT preserved) Lot 1 | Margolis & Duewer | A | 6 years | -80° C | Baseline | 0.1% |
| Ascorbic Acid (TAA , DTT preserved) Lot 2 | Margolis & Duewer | A | 6 years | -80° C | Baseline | 0.2% |
| Ascorbic Acid (TAA, MPA preserved) Lot 1 | Margolis & Duewer | A | 6 years | -80° C | Baseline | -1.1% |
| Ascorbic Acid (TAA, MPA preserved) Lot 2 | Margolis & Duewer | A | 6 years | -80° C | Baseline | -0.9% |
| Ascorbic Acid (MPA preserved) Lot 1 | Margolis & Duewer | A | 6 years | -80° C | Baseline | -4.1% |
| Ascorbic Acid (MPA preserved) Lot 2 | Margolis & Duewer | A | 6 years | -80° C | Baseline | -7.0% |
| Ascorbic Acid (no stabilization) | Jenab | A | 7 - 11 years | -196° C | Baseline | -3.3% - -2.1% |

| Table 8: B Vitamins | | | | | | |
|---|---|---|---|---|---|---|
| **Analyte** | **Study** | **Evidence Level** | **Storage Time** | **Storage Temp** | **Comparison Value** | **Annual % Change** |
| Folate | Jansen | A | 1 year | -196° C vs. -80° C vs. -20° C | Baseline | Stable at -80 & below -20 declined, but rank order same |
| Folate | Hustad | A | 29 years | -25° C | Baseline | -2.9% |
| Folate | Hannisdal | A | 29 years | -25° C | Baseline | -0.7% *retrieval method |
| Folate | Ocke | A | 4 years | -20° C | Baseline | -5.6% |
| Folate | Ihara | A | 1 year | -20° C | Baseline spiked | -.09% |
| B1 (Thiamine) | Ihara | A | 1 year | -20° C | Baseline spiked | 1.2% |
| B1 (Thiamine) | Ocke | A | 4 years | -20° C | Baseline | -0.8% |
| B2 (Riboflavin) | Ihara | A | 1 year | -20° C | Baseline spiked | -1.8% |
| B2 (Riboflavin) | Hustad | A | 29 years | -25° C | Baseline | 2.6% |
| B6 (Pyridoxine) | Ocke | A | 4 years | -20° C | Baseline | -0.9% |
| B6 (Pyridoxine) | Hustad | A | 29 years | -25° C | Baseline | -0.86% |
| B12 (Cyanocoblamin) | Ocke | A | 4 years | -20° C | Baseline | -3% |
| B12 (Cyanocoblamin) | Jansen | A | 1 year | -196° C vs. -80° C vs 20° | Baseline | Stable at all temperatures |
| B12 (Cyanocoblamin) | Hustad | A | 29 years | -25° C | Baseline | .35% |
| B12 (Cyanocoblamin) | Ocke | A | 4 years | -20° C | Baseline | 1.9% |

| Table 9: Steroid Hormones | | | | | | |
|---|---|---|---|---|---|---|
| Hormone | Study | Evidence Level | Storage Time | Storage Temp | Comparison Source | % Annual Change |
| Estradiol Premenopausal Serum | Bolelli | A | 3 years | -80° C | Baseline | -0.14% |
| Estradiol Postmenopausal Serum | Bolelli | A | 3 years | -80° C | Baseline | 0.23% |
| Estradiol Premenopausal Plasma | Bolelli | A | 3 years | -80° C | Baseline | -5.6% |
| Estradiol Postmenopausal Plasma | Bolelli | A | 3 years | -80° C | Baseline | -6.8% |
| Estradiol | Cauley | C | 10 years | -80° C | Fresh Samples | "Unchanged" |
| Estradiol | Barrett-Connor (90 & 95) | D | 15 years | -80° C | Published Results | "Remained Constant" |
| Estradiol | Garland | D | 14 years | -80° C | Unpublished Results | "Remained Constant" |
| Estradiol | Holl | D | 2-22 years | -25° C | Clinical Range | "Increased, but remained within clinically normal range" |
| Estradiol | Henderson | B/D ? | 21 years | -20° C | Samples over 6 years | "Stable" |
| Estradiol | Zhang | D | 40 years | -20° C | Published Results | "Stable" |

| Table 9 (cont'd) | | | | | | |
|---|---|---|---|---|---|---|
| Hormone | Study | Evidence Level | Storage Time | Storage Temp | Comparison Source | % Annual Change |
| Estiol | Zhang | D | 40 years | -20° C | Published Results | "Stable" |
| Estrone | Cauley | C | 10 years | -80° C | Fresh Samples | "Unchanged" |
| Estrone | Garland | D | 14 years | -80° C | Unpublished Results | "Remained Constant" |
| | | | | | | |
| Progesterone Premenopausal Serum | Bolelli | A | 3 years | -80° C | Baseline | -14.4% |
| Progesterone Premenopausal Plasma | Bolelli | A | 3 years | -80° C | Baseline | -13.1% |
| Progesterone | Holl | D | 2-22 years | -25° C | Clinical Range | "Decreased, but clinically normal range" |
| | | | | | | |
| Prolactin Premenopausal Serum | Bolelli | A | 3 years | -80° C | Baseline | 1.4% |
| Prolactin Postmenopausal Serum | Bolelli | A | 3 years | -80° C | Baseline | -1.4% |
| Prolactin Premenopausal Plasma | Bolelli | A | 3 years | -80° C | Baseline | 1.4% |
| Prolactin Postmenopausal Plasma | Bolelli | A | 3 years | -80° C | Baseline | 2.3% |
| | | | | | | |
| Total Testosterone Premenopausal Serum | Bolelli | A | 3 years | -80° C | Baseline | 3.4% |
| Total Testosterone Postmenopausal Serum | Bolelli | A | 3 years | -80° C | Baseline | -1.5% |

| Table 9 (cont'd) | | | | | | |
|---|---|---|---|---|---|---|
| Hormone | Study | Evidence Level | Storage Time | Storage Temp | Comparison Source | % Annual Change |
| Total Testosterone Premenopausal Serum | Bolelli | A | 3 years | -80° C | Baseline | 3.8% |
| Total Testosterone Postmenopausal Plasma | Bolelli | A | 3 years | -80° C | Baseline | 3.5% |
| Total Testosterone | Cauley | C | 10 years | -80° C | Fresh Samples | "Unchanged" |
| Total Testosterone | Garland | D | 14 years | -80° C | Unpublished Results | "Remained Constant" |
| Total Testosterone | Barrett-Connor (90 & 95) | D | 15 years | -80° C | Published Results | "Remained Constant" |
| Total Testosterone | Holl | D | 2-22 years | -25° C | Clinical Range | "Unchanged" |
| Total Testosterone | Henderson | B/D ? | 21 years | -20° C | Samples over 6 years | "Stable" |
| Total Testosterone | Stroud 3rd Trimester | D | 40 years | -20° C | Published Fresh Samples | 0.09% |
| Total Testosterone | Stroud Day of Delivery | D | 40 years | -20° C | Published Fresh Samples | -o.o8% |
| Total Testosterone | Zhang | D | 40 years | -20° C | Published Results | "Stable" |
| | | | | | | |
| Free Testosterone Premenopausal Serum | Bolelli | A | 3 years | -80° C | Baseline | 7.8% |
| Free Testosterone Premenopausal Serum | Bolelli | A | 3 years | -80° C | Baseline | 13.1% |
| Free Testosterone | Cauley | C | 10 years | -80° C | Fresh Samples | "Unchanged" |

| Table 9 (cont'd) | | | | | | |
|---|---|---|---|---|---|---|
| Hormone | Study | Evidence Level | Storage Time | Storage Temp | Comparison Source | % Annual Change |
| Cortisol | Stroud NCPP 3rd Trimester | C | 40 years | -20° C | Published Fresh Samples | -0.044% |
| Cortisol | Stroud NCPP Day of Delivery | C | 40 years | -20° C | Published Fresh Samples | -0.87% |
| | | | | | | |
| Androstenedione | Cauley | C | 10 years | -80° C | Fresh Samples | "Unchanged" |
| Androstenedione | Garland | D | 14 years | -80° C | Unpublished Results | "Remained Constant" |
| | | | | | | |
| Sex Hormone Binding Globulin | Barrett-Connor (1990) | D | 15 years | -80° C | Published Results | "Remained Constant" |
| Sex Hormone Binding Globin | Garland | D | 14 years | -80° C | Unpublished Results | "Remained Constant" |
| Sex Hormone Binding Globin | Holl | D | 2-22 years | -25° C | Clinical Range | "Increased, but remained within clinically normal range" |
| Sex Hormone Binding Globulin | Henderson | B | 21 years | -20° C | Samples over 6 years | "Stable" |
| Sex Hormone Binding Globulin | Lapidus | C/D | 16 years | -20° C | Fresh Samples & Published Results | "No Difference" |
| Sex Hormone Binding Globulin | Stroud NCPP 3rd Trimester | D | 40 years | -20° C | Published Fresh Samples | -0.44% |

| Table 9 (cont'd) | | | | | | |
|---|---|---|---|---|---|---|
| Hormone | Study | Evidence Level | Storage Time | Storage Temp | Comparison Source | % Annual Change |
| Sex Hormone Binding Globulin | Stroud NCPP Day of Delivery | D | 40 years | -20° C | Published Fresh Samples | -0.94% |

# REFERENCES

## REFERENCES


1. Agborsangaya, C., Toriola, A. T., Grankvist, K., Surcel, H., Holl, K., Parkkila, S., Tuohimaa, P., Lukanova, A., Lehtinen, M., The Effects of Storage Time and Sampling Season on the Stability of Serum 25-Hydroxy Vitamin D and Androstenedione, Nutrition and Cancer, 62(1) (2009) 51-57

2. Ambrosone C.B., Nesline M.K., Davis W., Establishing a Cancer Center Data Bank and Biorepository for Multidisciplinary Research, CEBP, 15(9) (2006) 1575-1577

3. Antoniucci, D.M., Black, D.M., Sellmeyer, D.E., Serum 25-Hydroxyvitamin D Is Uneffected by Multiple Freeze-Thaw Cycles, Clinical Chemistry, 50(1) (2005) p. 258-261

4. Barreto-Lins, M.H., Campos, F., Azevedo, M.C., Flores, H., A Re-Examination of the Stability of Retinol in Blood and Serum, and Effects of a Standardized Meal, Clin. Chem. 34(11) (1988) 2308-2310

5. Barrett-Connor E., Garland C., McPhillips J.B., Khaw K.T., Wingard D.L., A prospective, population-based study of androstenedione, estrogens, and prostatic cancer, Cancer Res. 50 (1990) 169-173

6. Barton R.H., Nicholson J.K., Elliott P., Holmes E., High-throughput H-NMR-based Metabolic Analysis of Human Serum and Urine for Large-scale Epidemiological Studies: Validation Study, Int J Epidemiol 37 (2008) i31-i40

7. Berg, B., Estborn, B., Tryding, N. Stability of serum and blood constituents during mail transport. Vol.41. Sweden: Medisinsk Fysiologisk Forenings Forlag; 1981. p.425-430.

8. Bolelli G., Muti P., Micheli A., Sciajno R., Franceschetti F., Krogh V., Pisani P., Berrino F., Validity for Epidemiological Studies of Long-Term Cryoconservation of Steroid and Protein Hormones in Serum and Plasma, Cancer Epidemiol 4 (July/August 1995) 509-513

9. Boyanton, B.L., Blick, K.E., Stability Studies of Twenty-Four Analytes in Human Plasma and Serum, Clinical Chemistry, 48(12) (2002) 2242-2247

10. Bradly, D.W., Emery, G., Maynard, J.E., Vitamin C in Plasma: A Comparative Study of the Vitamin Stabilized with Trichloroacetic Acid or Metaphosphoric Acid and the Effects of Storage at -70, -20, 4, and 25 on the Stabilized Vitamin. Clinica Chimica Acta, 40 (1973) 47-52

11. Brown, E.D., Micozzi, M.S., Craft, N.E., Bieri, J.G., Plasma Carotenoids in Normal Men After a Single Ingestion of Vegetables or Purified Beta-Carotene, Am J Clin Nutr 49 (1989) 1258-65

12. Bruning, P.F., Jonker, K.M, Boerema-Baan, A.W., Adsorption of Steroid Hormones by plastic tubing, J. Steroid Bio Chem, 14 (1981) 553-555

13. Cauley, J.A., Gutai, J.P., Huller, L.H., Dai, W.S., Usefulness of Sex Steroid Hormones in Predicting Coronary Artery Disease in Men, Am J Cardio, 60 (1987) 771-777

14. Clark, S., Youngman, L.D., Chukwurah, B., Palmer, A., Parish, S., Peto, R., Collins, R., Effect of Temperature and Light on the Stability of Fat-soluble Vitamins in Whole Blood Over Several Days: Implications for Epidemiological Studies, International Journal of Epidemiology 33 (2004) 518-525

15. Clark, S., Youngman, L.D., Palmer, A., Parish, S., Peto, R., Collins, R., Stability of Plasma Analytes after Delayed Separation of Whole Body: Implications for Epidemiological Studies, International Journal of Epidemiology 32 (2003) 125-130

16. Clevidence, B.A., Ballard-Barbash, R., Tocopherol Contents of Lipoproteins from Frozen Plasma Separated by Affinity Chromatography, Lipids 26 (1991) 723-728

17. Comstock G.W., Norkus E.P., Hoffman S.C., Xu M.W., Helzlsouer K.J., Stability of ascorbic acid, carotenoids, retinol, and tocopherols in plasma stored at -70 degrees C for 4 years, Cancer Epidemiol. Biomarkers Prev. 4(5) (1995) 505-507

18. Comstock G.W., Alberg A.J., Helzlsouer K.J., Reported effects of long-term freezer storage on concentrations of retinol, beta-carotene, and alpha-tocopherol in serum or plasma summarized, Clin. Chem. 39 (1993) 1075-1078

19. Craft, N.E., Brown, E.D., Smith, J.C., Effects of Storage and Handling Conditions on Concentrations of Individual Carotenoids, Retinol, and Tocopherol in Plasma, Clin Chem 34 (1988) 44-8

20. Downey P., Peakman T.C., Design and Implementation of a High-throughput Biological Sample Processing Facility using Modern Manufacturing Principles, Int J Epidemiol 37 (2008) i46-i50

21. Driskell, W.J., Lackey, A.D., Hewett, J.S., Bashor, M.M., Stability of Vitamin A in Frozen Sera, Clin. Chem. 31(6) (1985) 871-872

22. Dunn W.B., Broadhurst D., Ellis D.I., Brown M., Halsall A., O'Hagan S., Spasic I., Tseng A., Kell D.B., A GC-TOF-MS Study of the Stability of Serum and Urine Metabolomes During the UK Biobank Sample and Collection Protocols, Int J Epidemiol 37 (2008) i23-i30

23. Elliott P., Peakman T.C., The UK Biobank Sample Handling and Storage Protocol for the Collection, Processing and Archiving of Human Blood and Urine, Int J Epidemiol 37 (2008) 234-244

24. Fujita, M., Brindle, E., Shofer, J., Ndemwa, P. Kombe, Y., Shell-Duncan. B., O'Connor, K.A., Retinol-binding protein stability in dried blood spots, Clin Chem 53 (11) (Nov. 2007) 1972-1975

25. Garde A.H., Hansen A.M., Long-term stability of salivary cortisol, Scand. J. Clin. Lab. Invest. 65 (2005) 433-436

26. Garland, C.F., Friedlander, N.J., Barrett-Connor, E., Khaw, K., Sex Hormones and Postmenopausal Breast Cancer: A Prospective Study in an Adult Community, American Journal of Epidemiology 135(11) (1992) 1220-1230

27. Gunter, E.W., Driskell, W.J., Yeager, P.R., Stability of Vitamin E in Long-term Stored Serum, Chinica Chimica Acta 175 (1988) 329-336

28. Hankinson S.E., London S.J., Chute C.G., et al, Effect of transport conditions on the stability of biochemical markers in blood, Clin. Chem. 35 (1989) 2313-2316

29. Hannisdal, R., Gislefoss, R.E., Grimsrud, T.K., Hustad, S., Mokrid, L., Ueland, P.M., Analytical Recovery of Folate and Its Degradation Products in Human Serum Stored at -25C for up to 29 Years, J. Nutr. 140 (2010) 522-526

30. Hansen C, Shultz T. Nutritional Methodology-Research Communication: Stability of Vitamin B-6-Dependent Aminotransferase Activity in Frozen Packed Erythrocytes Is Dependent on Storage Temperature. The Journal of Nutrition; 2001. p. 1581- 1583.

31. Harman S.M., Metter E.J., Tobin J.D., Pearson J., Blackman M.R., Longitudinal effects of aging on serum total and free testosterone levels in healthy men, J. Clin. Endocrinol. Metab. 86 (2001) 724-731

32. Henderson B.E., The early in utero oestrogen and testosterone environment of blacks and whites: Potential effects of male offspring, Brit. J. Cancer 57 (1988) 216-218

33. Holl, K., Lundin, E., Kaasila, M., Grankvist, K., Afanasyeva, Y., Hallmans, G., Lehtinen, M., Pukkala, E., Surcel, H., Toniolo, P., Zeleniuch-Jacquotte, A., Koskela, P., Lukanova, A., Effect of Long-term Storage on Hormone Measurements in Samples from Pregnant Women: The experience of the Finnish Maternity Cohort, Acta Oncologica 47 (2008) 406-412

34. Holland N., Smith M., Eskenazi B., Bastaki M., Biological Sample Collection and Processing for Molecular Epidemiological Studies, Mutation Research 543 (2003) 217-234

35. Hustad, S., Eussen, S., Midttun, O., Ulvik, A., van de Kant, P.M., Morkid, L., Gislefoss, R., Ueland, P.M., Kinetic Modeling of Storage Effects on Biomarkers Related to B Vitamin Status and One-Carbon Metabolism, Clin. Chem. 58(2) (2012) 402-410

36. Ihara, H., Hashizume, N., Matsubayashi, T., Futaki, K., Yoshida, M., Sagawa, N., Fujisaki, M., Mita, K., Kadota, A., Stability of Fat-Soluble and Water-Soluble Vitamins in Artificially Prepared, Vitamin-Enriched, Lyophilized Serum, Journal of Clinical Laboratory Analysis 18 (2004) 240-246

37. Ito, Y., Wakai, K., Suzuki, K., Tamakoshi, A., Seki, N., Ando, M., Nishino, Y., Kondo, T., Watanabe, Y., Ozasa, K., Ohno, Y., Serum Carotenoids and Mortality from Lung Cancer: a case-control study nested in the Japan Collaborative Cohort (JACC) Study, Cancer Sci 94(1) (Jan 2003) 57-63

38. Ito, Y., Suzuki, K., Ichino, N., Imai, H., Sakaguchi, H., Hokama, M., Nishii, M., Nakano, H., The Risk of Helicobacter Pylori Infection and Atrophic Gastritis from Food and Drink Intake: a Cross-sectional Study in Hokkaido, Japan, Asian Pacific Journal of Cancer Prevention 1 (2000) 147-156

39. Jackson C., Best N., Elliott P., UK Biobank Pilot Study: Stability of Haematological and Clinical Chemistry Analytes, Int J Epidemiol 37 (2008) i16-i22

40. Jansen, E., Beekhof, P.K., Cremers, J., Schenk, E., Long-term (in)stability of folate and vitamin B12 in human serum, Clin Chem Lab Med 50(10) (2012) 1761-1763

41. Jenab M, Bingham S, Ferrari P, Friesen M, Al-Delaimy W, Luben R, Wareham N, Khaw K, Riboli E. Short Communication: Long-term Cryoconservation and Stability of Vitamin C in Serum Samples of the European Prospective Investigation into Cancer and Nutrition. Cancer Epidemiology, Biomarkers & Prevention; 2005. p. 1837-1840.

42. Kark, J.D., Smith, A.H., Switzer, B.R., Hames, C.G., Serum Vitamin A (Retinol) and Cancer Incidence in Evans County, Georgia, JNCI 66(1) (Jan 1981) 7-16

43. Karlsen, A., Biomhoff, R, Gundersen T.E., Stability of whole blood and plasma ascorbic acid, European Journal of Clinical Nutrition 61 (2007) 1233-1236

44. Kesner, J.S., Knecht, E.A., Krieg, E.F., Stability of Urinary Female Reproductive Hormones Stored Under Various Conditions, Reproductive Technology 9(3) (1995) 239-244

45. Key T.J., Pike M.C., Baron J.A., Moore J.W., Wang D.Y., Thomas B.S., Bulbrook R.D., Cigarette smoking and steroid hormones in women, J Steroid Biochem Mol Biol. 39(4A) (1991) 529-534

46. Konaromy-Hiller G., Nuttall K.L., Ashwood E.R., Effect of storage on serum vitamin B12 and folate stability, Ann. Clin. Lab. Sci. 27 (1997) 249-253

47. Landi M.T., Caporaso N., Sample collection, processing and storage, IARC Sci. Publ. (1997) 223-236

48. Lapidus, L., Lindstedt, G., Lundberg, P., Bengtsson, C., Gredmark, T., Concentrations of Sex-Hormone Binding Globulin and Corticosteroid Binding Globulin in Serum in Relation to Cardiovascular Risk Factors and to 12-Year Incidence of Cardiovascular Disease and Overall Mortality in Postmenopausal Women, Clin. Chem 32(1) (1986) 146-152

49. Lensmeyer, G.L., Wiebe, D.A., Binkley, N., Dreznerk, M.K., HPLC Method for 25-Hydroxyvitamin D Measurement: Comparison with Contemporary Assays, Clin. Chem 52(6) (2006) 1120-1126

50. Lippi, G., Chance, J.J., Church, S., Dazzi, P., Fontana, R., Glavarina, D., Grankvist, K., Huisman, W., Kouri, T., Palicka, V., Plebani, M., Puro, V., Salvagno, G.L., Sandberg, S., Sikaris, K., Watson, I., Stankovic, A.K., Simundic, A., Preanalytical Quality Improvement" from Dream to Reality; Clin. Chem 49(7) (2011) 1113-1126

51. Livesey, Hodgkinson, Roud. Stability of hormone during repeated freezing and thawing of plasma. p.152-155.

52. Manolio T.A., Biorepositories-at the Bleeding Edge, Int J Epidemiol 37 (2008) 231-233

53. Margolis, S.A., Davis, T.P., Stabilization of Ascorbic Acid in Human Plasma, and Its Liquid-Chromatographic Measurement, Clin. Chem 34(11) (1988) 2217-2223

54. Margolis, S.A., Duewer, D.L., Measurement of Ascorbic Acid in Human Plasma and Serum: Stability, Intralaboratory Repeatability, and Interlaboratory Reproducibility, Clin. Chem 42(8) (1996) 1257-1262

55. Margolis, S.A., Paule, R.C., Ziegler, R.G., Ascorbic and Dehydroascorbic Acids Measured in Plasma Preserved with Dithiothreitol or Metaphosphoric Acid, Clin. Chem 36(10) (1990) 1750-1755

56. Margolis, S.A., Ziegler, R.G., Helzlsouer, K.J., Ascorbic and Dehydroascorbic Acid Measurement in Human Serum and Plasma, Am J Clin Nutr 54 (1991) 1315S-18S

57. Maruvada, P., Srivastava, S., Biomarkers for Cancer Diagnosis: Implications for Nutritional Research, Journal of Nutrition (2004) 1640S-1645S

58. Mathew, G., Zwart, S.R., Smith, S.M., Stability of Blood Analytes After Storage in BD SST Tubes for 12 mo, Clinical Biochemistry 42 (2009) 1732-1734

59. Mathews-Roth, M.M., Plasma Concentrations of Carotenoids after Large Doses of Beta-Carotene, Am J Clin Nutr 52 (3) (1990) 500-1

60. Mathews-Roth M.M., Stampfer M.J., Some factors affecting determination of carotenoids in serum, Clin. Chem. 30 (1984) 459-461

61. McQuillan A.C., Sales S.D., Designing an Automated Blood Fractionation System, Int J Epidemiol 37 (2008) i51-i55

62. Miki K., Sudo A., Effect of urine pH, storage time, and temperature on stability of catecholamines, cortisol, and creatinine, Clin. Chem. 44 (1998) 1759-1762

63. Naito, M., Eguchi, H., Okada, R., Ishida, Y., Nishio, K., Hishida, A., Wakai, K., Tamakoshi, A., Hamajaima, N., Controls for Monitoring The Deterioration of Stored Blood Samples in the Japan Multi-Institutional Collaborative Cohort Study (J-MICC Study), Med. Sci. 70 (2008) 107-115

64. Nierenberg, D.W., Stukel, T.A., Baron, J.A., Dain, B.J., Greenberg, E.R., Determinants of Plasma Levels of Beta-Carotene and Retinol, American Journal of Epidemiology 130 (3) (1989) 511-521

65. Nomura, A.M.Y., Stemmermann, G.N., Helibrun, L.K., Salkeld, R.M., Vulleumier, J.P., Serum Vitamin Levels and the Risk of Cancer of Specific Sites in Men of Japanesse Ancestry in Hawaii, Cancer Research 45 (May 1985) 2369-2372.

66. Ockè M.C., Schrijver J., Obermann-de Boer G.L., Bloemberg B.P., Haenen G.R., Kromhout D., Stability of blood (pro)vitamins during four years of storage at -20 degrees C: consequences for epidemiologic research, J. Clin. Epidemiol. 48(8) (1995) 1077-85

67. Oddoze, C., Lombard, E., Portugal, H., Stability Study of 81 Analytes in Human Whole Blood, in Serum and in Plasma, Clinical Biochemistry 45 (2012) 464-469

68. Owen J.M., Woods P., Designing and Implementng a Large-scale Automated -80C Archive, Int J Epidemiol 37 (2008) i56-i61

69. Ozasa, K., Ito, Y., Suzuki, K., Watanbe, Y., Wakai, K., Tamakoshi, A., Association of Serum Carotenoid Concentration and Dietary Habits among the JACC Study Subjects, Journal of Epidemiology 15(2) (June 2005) S220-S227

70. Peakman T.C., Elliot P., The UK Biobank Sample Handling and Storage Validation Studies, Int J Epidemiol 37 (2008) i2-i6

71. Peng, Y., Xu, M., Alberts, D.S., Analysis and Stability of Retinol in Plasma, JNCI 78(1) (Jan 1987) 95- 99

72. Reyna, R., Traynor, K.D., Hines, G., Boots, L.R., Azziz, R., Repeated Freezing and Thawing does not Generally Alter Assay Results for Several Commonly Studied Reproductive Hormones, American Society for Reproductive Medicine 76(4) (Oct 2001) 823-825

73. Riboli, E., Hunt, K.J., Slimani, N., Ferrari, P., Norat, T., Fahey, M., Charrondiere, U.R., Hemon, B., Casagrande, C., Vignat, J., Overvad, K., Tionneland, A., Clavel-Chapelon, G., Thiebaut, A., et.al., European Prospective Investigation into Cancer and Nutrition (EPIC): study populations and data collection, Public Health Nutr 5(6B) (Dec 2002) 113-24

74. Rider, A.A, Foard, M.A., Sommer, A., Studies on the Stability of Vitamin A and Related Compounds in Blood Subjected to Adverse Conditions Prior to Serum Seperation, Nutr Rep Int 29 (1984) 1307-14

75. Rundle, A.G., Vineis, P., Ahsan, H., Design Options for Molecular Epidemiology Research within Cohort Studies, Cancer Epidemiol Biomarkers Prev 14(8) (Aug 2005) 1899-1907

76. Smith, A.H., Waller, K.D., Serum Beta-Carotene in Persons with Cancer and their Immediate Familes, Am J Epidemiol 133 (1991) 661-71

77. Stroud LR, Solomon C, Shenassa E, Papandonatos G, Niaura R, Lipsitt LP, Lewinn K, Buka SL, Long-term stability of maternal prenatal steroid hormones from the National Collaborative Perinatal Project: still valid after all these years, Psychoneuroendocrinology 32(2) (Feb 2007) 140-150

78. Stryker, W.S., Stampfer, M.J., Stein, E.A., Kaplan, L., Louis, T.A., Sober, A., Willett, W.C., Diet, Plasma Levels of Beta-Carotene and Alpha-Tocopherol, and Risk of Malignant Melanoma, American Journal of Epidemiology 131(4) (1990) 597-611

79. Tamakoshi, A., Yoshimura, T., Inaba, Y., Ito, Y., Watanabe, Y., Fukuda, K., Isa, H., Profile of the JACC Study, Journal of Epidemiology 15(1) (Mar 2005) S4-S8

80. Tangney, C.C., Shekelle, R.B., Raynor, W., Gale, M., Betz, E., Intra- and Interindividual Variation in Measurements of beta-carotene, retinol, and tocopherols in diet and plasma, Am J Clin Nutr 45 (1987) 764-9

81. Tanner M., Kent N., Smith B., Fletcher S., Lewer M. Stability of common biochemical analytes in serum gel tubes subjected to various storage temperatures and times pre-centrifugation. Vol.45. Annals of Clinical Biochemistry; 2008. p.375-379.

82. Thumham, D.I., Smith, E., Flora, P.S., Concurrent Liquid Chromatographic Assay of Retinol, alpha –Tocopherol, beta- Carotene, alpha-Carotene, Lycopene, and beta-Cryptoxanthin in Plasma, with Tocopherol Acetate as Internal Standard, Clinical Chemistry 34(2) (1988) 377-381

83. Tworoger S.S., Hankinson S.E., Collection, processing, and storage of biological samples in epidemiologic studies: sex hormones, carotenoids, inflammatory markers,and proteomics as examples, Cancer Epidemiol. Biomarkers Prev. 15(9) (2006) 1578-81

84. Tworoger S.S., Hankinson S.E., Use of Biomarkers in Epidemiologic Studies: Minimizing the Influence of Measurement Error in the Study Design and Analysis, Cancer Causes Control 17 (2006) 889-899

85. Vaught J.B., Blood Collection, Shipment, Processing, and Storage, CEBP, 15(9) (2006) 1582-1584

86. Willett, W.C., Polk, B.F., Underwood, B.A., Stamper, M.J., Pressel, S., Rosner, B., Taylor, J.O., Schneider, K., Hames, C.G., Relation of Serum Vitamins A and E and Carotenoids to the Risk of Cancer, New England Journal of Medicine (Feb 1984) 430-434

87. Winn D.M., Reichman M.E., Gunter E., Epidemiologic issues in the design and use of biologic specimen banks, Epidemio. Rev., 12 (1990) 56-70

88. Zhang Y., Graubard B.I., Longnecker M.P., Stanczyk F.Z., Klebanoff M.A., McGlynn K.A., Maternal hormone levels and perinatal characteristics: implications for testicular cancer, Ann. Epidemiol. 17(2) (2007) 85-92